Chapter 3

Stimulatory effects of novel glucosylated lactose derivatives GL34 on growth of selected gut bacteria

Hien T. T. Pham, Markus C.L. Böger, Lubbert Dijkhuizen*, and Sander S. van Leeuwen

Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

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ABSTRACT

Previously we structurally characterized 5 Glucosylated Lactose derivatives (F1-F5) with a degree of polymerization (DP) of 3-4 (GL34), products of *Lactobacillus reuteri* glucansucrases, with lactose and sucrose as substrates. Here we show that these GL34 compounds are largely resistant to the hydrolytic activities of common carbohydrate degrading enzymes. Also the ability of single strains of gut bacteria, bifidobacteria, lactobacilli and commensal bacteria, to ferment the GL34 compounds was studied. Bifidobacteria clearly grew better on the GL34 mixture than lactobacilli and commensal bacteria. Lactobacilli and the commensal bacteria *Escherichia coli* Nissle and *Bacteroides thetaiotaomicron* only degraded the F2 compound α-D-Glc*p*-(1→2)-[β-D-Gal*p*-(1→4)-]D-Glc*p*, constituting around 30 % w w^{-1} of GL34. Bifidobacteria digested more than one compound from the GL34 mixture, varying with the specific strain tested. *Bifidobacterium adolescentis* was most effective, completely degrading four of the five GL34 compounds, leaving only one minor constituent. GL34 thus represents a novel oligosaccharide mixture with (potential) synbiotic properties toward *B. adolescentis*, synthesized from cheap and abundantly available lactose and sucrose.

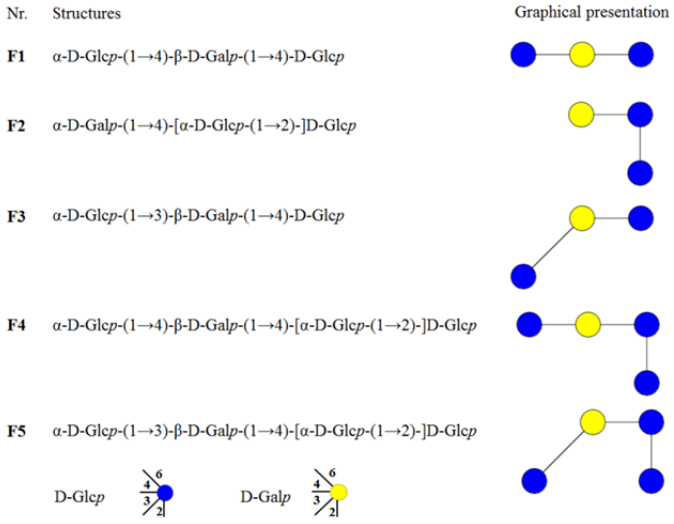
INTRODUCTION

The human gut microflora has drawn increasing attention in recent years. It constitutes a very interesting ecosystem that varies in density and functionality in the different gut compartments.[1] These complex ecosystems have a significant impact on host well-being.[2] Strongest interest is focused on understanding what factors cause variations in microbiota composition and how these gut bacteria modulate host health.[3] Our work aims to stimulate the growth of health-promoting probiotic gut bacteria by using newly synthesized non-digestible carbohydrates, i.e. prebiotic compounds.

According to the latest definition, a prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit".[4] Recently, part of the definition was disputed, since selective stimulation of health-promoting species seems not exclusively necessary to confer health benefits.[5,6] Generally, prebiotics are carbohydrates that are not fully digested by the host. They are fermented by various commensal and health-beneficial gut bacteria, thus promoting their growth and activity which may confer health benefits upon the host.[7,8] To date, the most well-known prebiotics, supported by good quality data, are human milk oligosaccharides (*h*MOS),[9] GOS (β-galacto-oligosaccharides), FOS (β-fructo-oligosaccharides), inulin and lactulose.[10,11] All of these prebiotics are also hydrolysed by brush border enzymes, but not completely.[12] Isomalto-/malto-polysaccharides (IMMP),[13,14] xylo-oligosaccharides (XOS),[15] resistant starch,[16] and soy oligosaccharides also are (emerging) prebiotic oligosaccharides,[17] although more data about their effects on gut health are still needed. Each of these prebiotic compounds may exert specific and selective effects on gut bacteria. The search for new and effective prebiotics combined with specific probiotics (synbiotics) is increasing rapidly.[18,19]

Lactose-derived oligosaccharides attract much attention in view of their prebiotic potential. One example is GOS, which are synthesized from lactose by enzymatic

trans-galactosylation using β-galactosidases, achieving a degree of polymerization between 3 and 10.[20] This prebiotic has been widely studied and shown to stimulate probiotic bacteria to various extents.[12,21,22] Another commercially available prebiotic in this group is lactosucrose which is hardly utilized by human digestive enzymes and has stimulatory effects on both lactobacilli and bifidobacteria.[23,24] Also the selective bifidogenic effect of 4'-galactosyl-kojibiose, corresponding to compound F2 in our GL34 mixture,[25] on *Bifidobacterium breve* 26M2 has been reported.[24] These results indicate that there are clear perspectives to further develop and expand this group of lactose-derived prebiotic oligosaccharides.



Scheme 1: Structures of compounds F1-F5 from the mixture GL34.

We recently reported synthesis of a mixture of five novel lactose-derived oligosaccharides (F1-F5) using the *L. reuteri* glucansucrase enzymes Gtf180-ΔN and GtfA-ΔN.[25] Their structural characterization revealed the presence of various glycosidic linkages, (α1→2/3/4), with DP of 3 and 4 (Scheme 1).[25] Four out of these five structures were new and only F2 4'-galactosyl-kojibiose had been reported before. In this work, their resistance to degradation by common carbohydrate

degrading enzymes was studied by *in vitro* incubations. Also the growth of pure cultures of common gut bacteria, including commensal and probiotic strains, on these novel compounds was evaluated and compared with well-known prebiotic mixtures (GOS and FOS). This study provides information about the selective stimulatory effects of these compounds (and glycosidic linkage types) on growth of probiotic bacteria. The GL34 mixture particularly stimulated growth of *B. adolescentis*. This is also of interest from an industrial perspective, since these new oligosaccharides with very specific prebiotic effects are produced from low-cost lactose and sucrose, and may be an option for developing synbiotics.

MATERIALS AND METHODS

Bacterial strains, chemicals and reagents

Bacteroides thetaiotaomicron VPI-5482, *Lactobacillus acidophilus* ATCC 4356, *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium longum* subsp. *infantis* ATCC 15697 were purchased from ATCC. *Bifidobacterium breve* DSM 20213 was obtained from the DSMZ culture collection. *Lactobacillus reuteri* strain 121 was obtained from TNO Quality of Life, Zeist, The Netherlands. *Lactobacillus casei* W56 was provided by Winclove Probiotics B.V. (Amsterdam). All reagents, chemicals or medium components were purchased from Sigma (Zwijndrecht, Netherlands), or as stated otherwise**.** The purified GOS mixture TS0903 (lacking glucose, galactose and lactose) was provided by FrieslandCampina Domo; its detailed GOS composition was published elsewhere.[26] The GOS/FOS mixture used is a 90/10 (w w^{-1}) mixture of the purified TS0903 GOS and long-chain Inulin (lcInulin, Frutafit TEX, provided by SENSUS, Roosendaal, The Netherlands), also serving as a control for the current prebiotic formula added to infant nutrition.[21]

Lactobacillus growth experiments

Lactobacilli were pre-cultured in MRS-medium (Oxoid, Basingstoke, UK) anaerobically (or by using the GasPak system (Becton, Dickinson and Company, Sparks, USA)) under an N_2 atmosphere for up to 2 days at 37°C.[27] Then, 1 ml samples of the pre-cultures were harvested by centrifugation (2,500 x g, 2 min). The bacterial pellets were washed twice with sterile 10% NaCl and diluted 25-fold in 2x mMRS (modified MRS-medium that does not contain a carbon source for Lactobacilli).[28] In separate tubes, carbohydrates were dissolved with Milli-Q water to 10 mg ml^{-1} and sterilized by filtration using 0.2 μm cellulose acetate filters (the GL34 mixture) or by autoclaving solutions (glucose). Cultures were inoculated by mixing 1:1 diluted bacterial suspensions with sterilized carbohydrate solutions in sterile microtiter plates to obtain an initial OD_{600} of 0.01. Inoculation of microtiter plates was carried out in an anaerobic glove box (Sicco, Grünsfeld, Germany) with a constant nitrogen-flow, the microtiter plates sealed air tightly and transferred into a plate reader placed under constant N_2 flow. Glucose was used as positive control to compare growth of these lactobacilli on the mixture of GL34 compounds. Media without an added carbon source was used as negative control. Bacterial growth was followed at 37 °C by measuring the optical density at 600 nm (OD_{600} nm) in 5 min intervals. OD values of the negative control samples (no carbohydrate added) were deducted while measuring their corresponding samples and positive controls.

Bifidobacterium and *B. thetaiotaomicron* growth experiments

The *Bifidobacterium* strains were sub-cultured (from stocks stored at −80 °C) in 10 mL of *Bifidobacterium* medium (BM) supplemented with 1% glucose (One liter BM contained 10 g Trypticase peptone, 2.5 g yeast extract, 3 g tryptose, 3 g K_2HPO_4, 3 g KH_2PO_4, 2 g triammonium citrate, 0.3 g pyruvic acid, 1 ml Tween 80, 0.574 g $MgSO_4$. $7H_2O$, 0.12 g $MnSO_4$. H_2O, and 0.034 g $FeSO_4$. $7H_2O$). After autoclaving, BM was supplemented with 0.05 % (wt vol^{-1}) filter-sterilized cysteine-HCl (Ryan et

al. 2006). *B. thetaiotaomicron* was cultured using a carbon-limited minimally defined medium of 100 mM KH_2PO_4 (pH 7.2), 15 mM NaCl, 8.5 mM $(NH_4)_2SO_4$, 4 mM L-cysteine, 1.9 M hematin, 200 M L-histidine, 100 nM $MgCl_2$, 1.4 nM $FeSO_4 \cdot 7\ H_2O$, 50 M $CaCl_2$, 1 g mL^{-1} vitamin K3, 5 ng mL^{-1} vitamin B12, and individual carbon sources (0.5%, wt vol^{-1}).[29] Carbohydrates were prepared by dissolving in Milli-Q water to 10 mg ml^{-1} and sterilized by filtration using 0.2 μm cellulose acetate filters (the GL34 mixture, GOS, FOS) or by autoclaving solutions (lactose). Growth was carried out in fermentation tubes at 37 °C under anaerobic conditions maintained by GasPak EZ anaerobe container system (BD, New Jersey, US). Cell suspensions from overnight cultures were prepared in 3 mL of BM supplemented with different carbon source in a final concentration of 5 mg mL^{-1}. BM without an added carbon source was used as negative control. The initial OD_{600} of all incubations was adjusted to 0.01. OD_{600} nm measurements of the fermentation tubes were carried out manually at 1-h intervals and data used to generate growth curves. OD values of the negative control samples (no carbohydrate added) were deducted while measuring their corresponding samples and positive controls.

E.coli Nissle growth experiments

E.coli Nissle was cultured using M9 medium as previously described, at 37 ^oC under aerobic conditions.[30] OD_{600} nm measurements of the fermentation tubes were carried out manually at 2-h intervals. OD values of the negative control samples (no carbohydrate added) were deducted while measuring their corresponding samples and positive controls.

Enzyme incubations

The GL34 mixture (1 mg mL^{-1}) was incubated for 24 h with different carbohydrate degrading enzymes (5 U mL^{-1}); α-amylase 1 (Porcine pancreas); α-amylase 2 (*Aspergillus oryzae*); α-glucosidase (Yeast); Iso-amylase (*Pseudomonas sp*.;

Pullulanase type 1 (*Klebsiella planticola*); β-galactosidase 1 (*Aspergillus oryzae*) and β-galactosidase 2 (*Kluyveromyces lactis*) (see Table S1 with detailed information).

Intracellular and extracellular activity essays

After growth with GL34 as their only carbon source, the three tested *Bifidobacterium* strains were harvested by centrifugation at 10,000 x g for 15 min at room temperature. Culture supernatants were sterilized using 0.45 µm filters and concentrated 10 times by Amicon Ultra-4 centrifugal filter units (10,000 Da molecular weight cutoff, Millipore). The harvested cell pellets were washed twice with 0.1 M potassium phosphate buffer (pH 6.6) and then suspended in 1 mL of this buffer into 2.0-mL screw-cap microtubes containing 400 mg of 0.1-mm-diameter glass beads (Biospec Products). Cell disruptions were carried out by homogenization by a mini bead-beater (Biospec Products) at 4,200 rpm for six 1-min cycles with 40 sec cooling on ice in between. The cytoplasmic extracts were harvested by centrifugation at 10,000 x g for 5 min to remove cell wall fragments, and then concentrated to one-fifth of the initial volume using Amicon Ultra-4 units as above.

The concentrated cell free supernatants and cytoplasmic extracts (10 µg protein for each) were incubated separately with 5 mg mL^{-1} of the GL34 mixture. All reactions were performed in Milli-Q at 37 °C for 24 h. The progress of the reactions was followed by high-performance-anion-exchange chromatography (HPAEC).

High-pH Anion-Exchange Chromatography (HPAEC)

Samples were analyzed on an ICS-3000 workstation (Dionex, Amsterdam, the Netherlands) equipped with an ICS-3000 pulse amperometric detection (PAD) system and a CarboPac PA-1 column (250 x 2 mm; Dionex). The analytical separation was performed at a flow rate of 0.25 mL min^{-1} using a complex gradient

of eluents A (100 mM NaOH); B (600 mM NaOAc in 100 mM NaOH); C (Milli-Q water); and D: 50 mM NaOAc. The gradient started with 10 % A, 85 % C, and 5 % D in 25 min to 40 % A, 10 % C, and 50 % D, followed by a 35-min gradient to 75 % A, 25 % B, directly followed by 5 min washing with 100 % B and reconditioning for 7 min with 10 % A, 85 % B, and 5 % D. External standards of lactose, glucose, fructose were used for calibration. For the determination of glucosylated lactose compounds with a degree of polymerization (DP) of 3, maltotriose was used as external standard.

Bioinformatic analysis

All protein sequences from *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis* ATCC 15697, *B. breve* DSM 20123 and *B. breve* UCC 2003 used in this study were extracted from the National Center for Biotechnology Information (NCBI) database. Database searches used the non-redundant sequence database accessible at the NCBI website (http://www.ncbi.nlm.nih.gov) using BLASTP and global align search. The BLASTP searches and multiple-sequence alignments were used to find similarity between the characterized glucosidases of *B. breve* UCC 2003 and annotated glucosidases encoded by the studied bifidobacterial strains. Annotation of Carbohydrate-active enzymes encoded by the genome sequences of *L. reuteri* 121 and *L. acidophilus* ATCC 4356 was carried out using dbCAN (http://csbl.bmb.uga.edu/dbCAN).

RESULTS

Enzymatic hydrolysis of compounds in the GL34 mixture

The GL34 mixture of five compounds was synthesized using glucansucrase Gtf180-ΔN, decorating lactose with one or two glucose units from sucrose as donor substrate, also introducing different types of linkages (Pham et al. 2017).[25] GL34

contains three DP3 compounds and two DP4 compounds, i.e. **F1** (4′-glc-lac): α-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc*p*; **F2** (2-glc-lac): α-D-Glc*p*-(1→2)-[β-D-Gal*p*-(1→4)-]D-Glc*p*; **F3** (3′-glc-lac): α-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*; **F4** (4′,2-glc-lac): α-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-[α-D-Glc*p*-(1→2)-]D-Glc*p* and **F5** (3′,2-glc-lac): α-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→4)-[α-D-Glc*p*-(1→2)-]D-Glc*p*.[25] Four types of glycosidic linkages thus occur in this mixture, namely (α1→2), (α1→3), (α1→4), and (β1→4). Only the F2 2-glc-lac compound had been described before.[31] The GL34 mixture also contains glucosyl residues linked (α1→3)/ (α1→4) to the galactosyl residue of the original lactose. In view of the novel composition of this mixture of glucosylated-lactose compounds we tested their resistance or sensitivity to hydrolysis with several commercially available enzymes. Following incubations with the porcine pancreas and *Aspergillus oryzae* α-amylases (Table S1), the HPAEC profiles at time 0 and 24 h showed no degradation of the GL34 compounds (Figure 1). Also various malto-oligosaccharide acting enzymes (α-glucosidase, iso-amylase and pullulanase, Table S1) were tested for their ability to hydrolyze GL34 compounds. However, after 24 h incubation, no (monomeric or dimeric) products were detected in the reaction mixtures with these three enzymes (Figure 1). None of these α-glucose cleaving enzymes thus was active on the GL34 compounds.

Subsequent incubation of the GL34 mixture with the β-galactosidase enzymes from *A. oryzae* and *Kluyveromyces lactis* (Table S1) however, did result in (some) hydrolysis. Fig. 1 shows that galactose and kojibiose (a glucose disaccharide with (α1→2)-linkage) were released during incubation with β-galactosidase, especially with the *A. oryzae* enzyme. Only the peak corresponding to F2 2-glc-lac disappeared, the only GL34 compound with a terminal galactosyl residue. We subsequently studied the utilization of these GL34 compounds for growth by (selected) common intestinal bacteria in more detail.

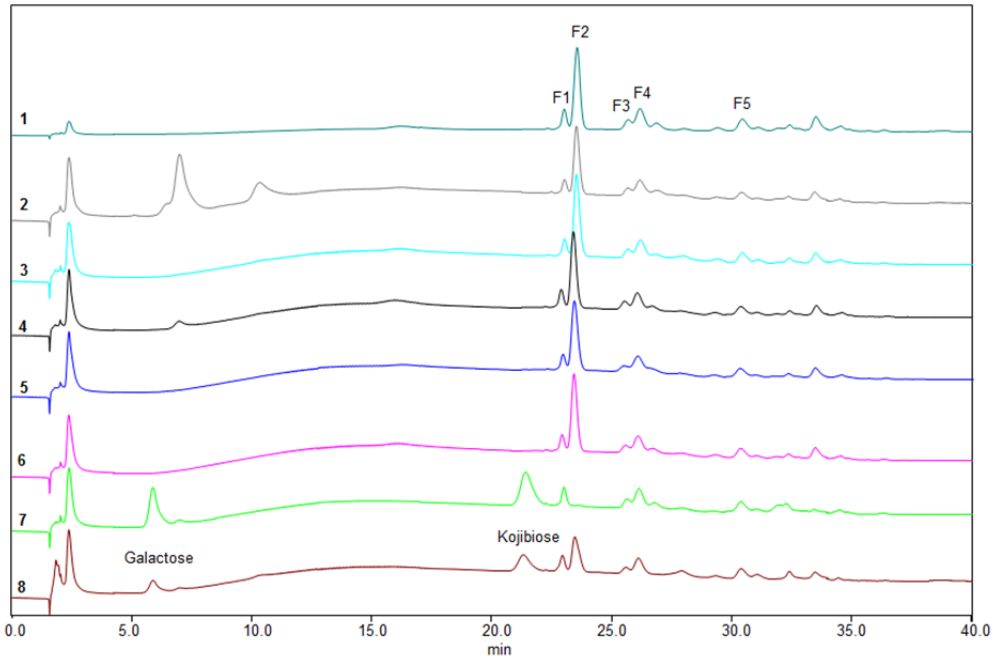


Figure 1: HPAEC profiles of oligosaccharides in 1) the GL34 mixture (1 mg mL^{-1}, blank) and the hydrolysis products after incubation of GL34 with 2) α-amylase from Porcine; 3) α-amylase from *A. oryzae*; 4) α-glucosidase from yeast; 5) iso-amylase from *Pseudomonas* sp.; 6) pullulanase type 1 from *K. planticola*; 7) β-galactosidase from *A. oryzae* and 8) β-galactosidase from *K. lactis*.

Growth of human gut bacteria on the GL34 mixture

Fermentation of GL34 compounds by probiotic Lactobacillus strains

In this study we tested *L. casei* W56, *L. acidophilus* ATCC 4356 and *L. reuteri* 121 and observed that all three strains showed limited growth on media with the GL34 mixture as the only carbon source (5 mg mL^{-1}): the final (relative) OD_{600} values reached were 3.8 %, 10.4 % and 26.5 %, respectively, compared to a 100 % control grown on glucose (Figure 2).

The GL34 mixture (5 mg mL^{-1}) showed different stimulatory effects on the growth of *B. breve* DSM 20123, *B. adolescentis* ATCC 15703 and *B. longum* subsp. *infantis* ATCC 15697. *B. adolescentis* grew very well on GL34, its final OD_{600} value

reached 80 % of that of a 100 % control growing on lactose, the purified TS0903 GOS mixture and the GOS/FOS mixture (Table 1). However, the final OD_{600} values for growth of *B. breve* DSM 20123 and *B. longum* subsp. *infantis* ATCC 15697 on GL34 remained below 50% of the values for growth on lactose, the purified TS0903 GOS mixture and the GOS/FOS mixture (Table 1).

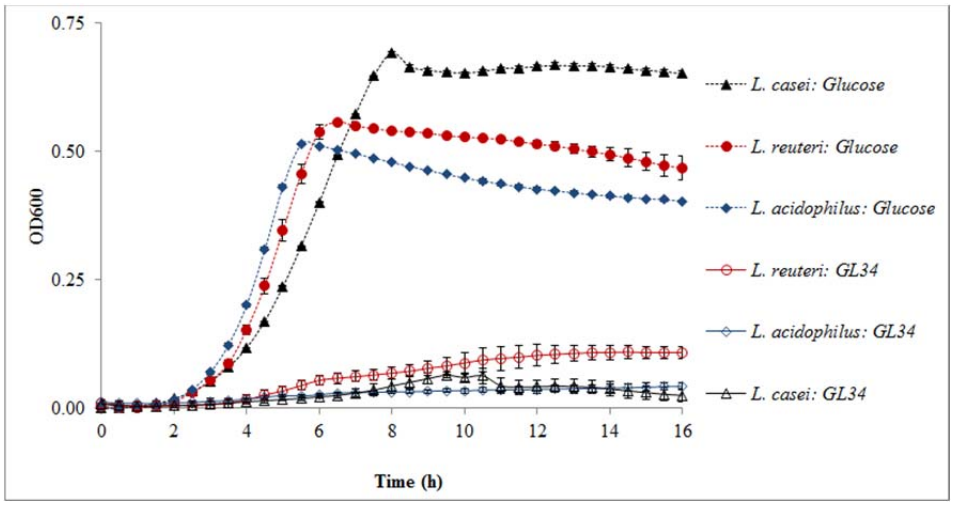


Figure 2: Growth of *L. casei* W56, *L. reuteri* 121 and *L. acidophilus* ATCC 4356 on GL34 compounds (5 mg mL^{-1}). Glucose (5 mg mL^{-1}) served as positive control; growth studies were carried out in triplicate.

Fermentation of GL34 compounds by probiotic Bifidobacterium strains

The tested bifidobacterial strains displayed two or more growth phases (Figure 3 and Figure S1). Some compounds in the GL34 mixture thus are more preferred growth substrates than others. *B. breve* and *B. longum* subsp. *infantis* grew more slowly on GL34 than *B. adolescentis*. They reached OD values around 0.70 after 24 h incubation compared to 12 h for *B. adolescentis*. Final maximal OD values were 0.86 for *B. breve* and 0.73 for *B. longum* subsp. *infantis* and 1.60 for *B. adolescentis.* The latter strain appeared to go through different lag phases, adapting to the

different carbon sources in GL34, reaching maximal OD after 36 h of incubation (Figure S1).

Table 1: Growth of *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis* ATCC 15697 and *B. breve* DSM 20123 on different carbon sources (5 mg mL^{-1}) for 36 h, and the final pH values.

Carbon sources	*Bifidobacterium adolescentis* ATCC 15703			*Bifidobacterium longum* subsp. *infantis* ATCC15697			*Bifidobacterium breve* DSM 20213		
	OD_{600}	%	pH	OD_{600}	%	pH	OD_{600}	%	pH
Lactose	2.00 ± 0.00	100	5.0	2.00 ± 0.00	100	5.0	2.00 ± 0.00	100	5.0
GOS	2.00 ± 0.00	100	5.0	1.77 ± 0.02	89	5.2	1.64 ± 0.04	82	5.3
GOS/FOS	2.00 ± 0.00	100	4.9	1.79 ± 0.02	90	5.3	1.61 ± 0.01	81	5.2
GL34	1.60 ± 0.03	80	5.0	0.73 ± 0.03	37	5.3	0.86 ± 0.04	43	5.4

The experiments were carried out in triplicate, and the average values are shown.

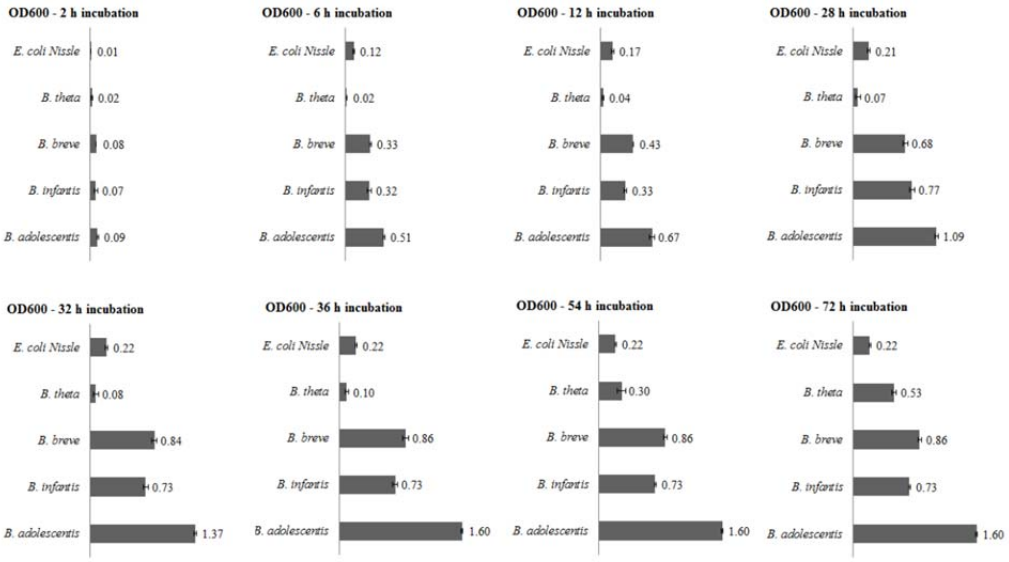


Figure 3: Growth of *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis* ATCC 15697, *B. breve* DSM 20123, *E. coli* Nissle, *B. thetaiotaomicron* in medium supplemented with 5 mg mL^{-1} GL34 at different times of incubation (h). Growth experiments were carried out in triplicate.

Fermentation of GL34 compounds by commensal gut bacteria

Also the ability of two selected commensal bacteria to grow on the GL34 mixture was studied. *B. thetaiotaomicron* is a Gram-negative anaerobic bacterium found dominantly in human distal intestinal microbiota.[32] *E.coli* Nissle represents ecologically important inhabitants of the human intestinal tract.[33] Growth of *E. coli* Nissle on the GL34 mixture, TS0903 GOS and a GOS/FOS mixture (5 mg mL^{-1}), was relatively minor with final OD_{600} values of 0.19 ÷ 0.35 after 24 h of incubation, compared to growth on lactose and glucose with final OD_{600} values of 0.66 and 0.74, respectively (Figure 4-1). The final OD_{600} value of *B. thetaiotaomicron* was 0.53 after 72 h of incubation, but its growth displayed a pronounced lag-phase (Figure 4-2). This strain thus may fail to compete with other bacteria which have shorter lag-phases of growth with the GL34 mixture, such as the bifidobacteria tested and *E. coli* Nissle.

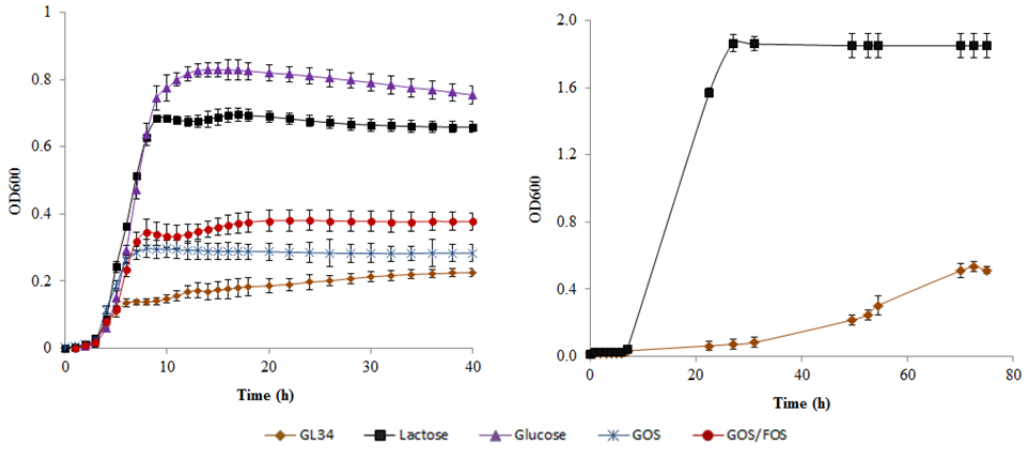


Figure 4: 1) Growth of *E.coli* Nissle on the GL34, TS0903 GOS and GOS/FOS mixtures (5 mg mL^{-1}); lactose and glucose (5 mg mL^{-1}) served as positive controls, and 2) Growth of *B. thetaiotaomicron* on the GL34 compounds (5 mg mL^{-1}); lactose (5 mg mL^{-1}) served as positive control. Growth studies were carried out in triplicate.

We subsequently identified the specific GL34 compounds utilized by these strains, and products derived, also aiming to elucidate which hydrolytic enzyme activities are involved, with emphasis on β-galactosidases and α-glucosidases.

Hydrolytic activity of commensal bacteria and lactobacilli on the GL34 mixture

The GL34 compounds can be visualized as individual peaks in HPAEC-PAD chromatograms. The compounds consumed by the tested bacteria were validated by peak disappearance at the corresponding retention time. HPAEC analysis of culture supernatants of the commensal bacteria grown on the GL34 mixture showed that the F2 peak corresponding to 2-glc-lac disappeared completely (Figure 5), indicating the selective and full utilization of only F2. Most likely this is based on β-galactosidase degradation of F2 2-glc-lac, followed by α-glucosidase degradation of the kojibiose formed, and finally consumption of the galactose and glucose formed for cellular growth. Only in case of *E. coli* Nissle degradation resulted in accumulation of kojibiose. This was not investigated any further. Besides, *B. thetaiotaomicron* was also able to degrade F4 4´,2-glc-lac partially (Figure 5).

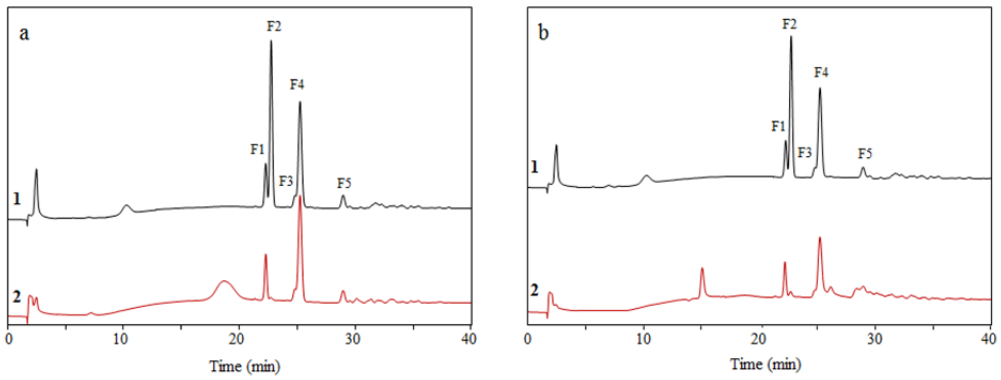


Figure 5: HPAEC profiles of oligosaccharides in **1**) the GL34 mixture (1 mg mL^{-1}, blank) and **2**) the hydrolysis products of GL34 fermentation by **a**) *E. coli* Nissle at 40 h and **b**) *B. thetaiotaomicron* at 72 h.

HPAEC profiles of *L. casei* W56 culture supernatants showed that none of the GL34 compounds were degraded (Figure 6), in line with the very limited growth observed (Figure 2). When searching the annotated genome sequence of this strain in the CAZy database (http://www.cazy.org/b7858.html) we did not find any (putative) β-galactosidase. This most likely explains the inability of *L. casei* W56 to hydrolyze the galactose (β1→4) linked to glucosyl residue in F2 2-glc-lac. Many putative α-glucosidases were found encoded in the genome sequence of this strain, however. In view of the results obtained, these enzymes apparently are inactive on the GL34 compounds, or the GL34 mixture is unable to induce their expression. This was not investigated further.

L. acidophilus ATCC 4356 and *L. reuteri* 121 strains only showed hydrolytic activity with F2 2-glc-lac in the GL34 mixture. *L. reuteri* 121 degraded this compound to a higher level than *L. acidophilus* ATCC 4356 (Figure 6). Annotation of Carbohydrate-active enzymes encoded by the genome sequence of *L. reuteri* 121 was carried out using dbCAN (http://csbl.bmb.uga.edu/dbCAN).[34] One putative β-galactosidase of family GH2 was detected (gene number: BJI45_06415), which may be responsible for hydrolysis of F2. β-Galactosidases of this GH2 family are known to hydrolyze a wide variety of β-(1→2, 3, 4, or 6) GOS, including oligosaccharides with a degree of polymerization of 3 – 6.[35] Kojibiose released from the F2 compound by this strain remained in the medium without being degraded (completely). Only a single (predicted) extracellular α-glucosidase of family GH31 was encoded in the genome of *L. reuteri* 121 (gene number: BIJ45_02455).[34] This enzyme is apparently unable to effectively degrade kojibiose. Apparently, there is also no transporter expressed that can facilitate the uptake of kojibiose for intracellular degradation.

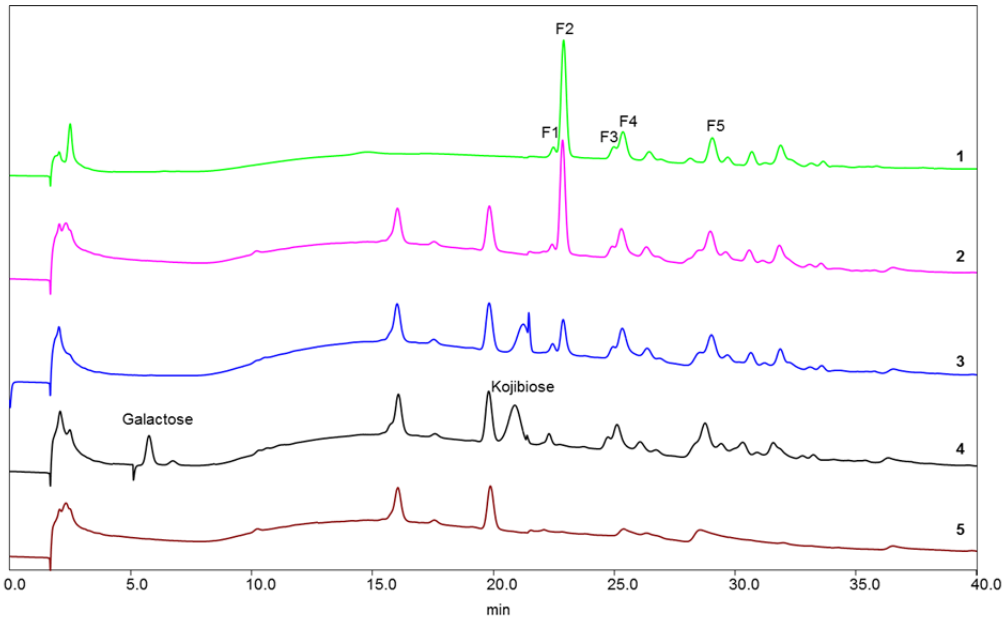


Figure 6: HPAEC profiles of oligosaccharides in 1) the GL34 mixture (1 mg mL^{-1}, blank) and the hydrolysis products of GL34 fermentation after 16 h of incubation with 2) *L. casei* W56; 3) *L. acidophilus* ATCC 4356; 4) *L. reuteri* 121 and 5) MRS medium (blank).

L. acidophilus ATCC 4356 is known to encode a β-galactosidase (LacZ, classified in the GH2 family).[35-37] This LacZ enzyme thus may be responsible for degradation of the (β1→4)-linkage in F2 and release of kojibiose. Annotation of Carbohydrate-active enzymes encoded by the genome sequence of *L. acidophilus* ATCC 4356 was also carried out by dbCAN (http://csbl.bmb.uga.edu/dbCAN).[38] Ten putative α-glucosidase enzymes (Family GH13 and GH31) were annotated in the *L. acidophilus* ATCC 4356 genome. Agl3 is an α-glucosidase identified in *B. breve* UCC 2003, with a broad hydrolytic activity towards all possible α-glycosidic linkages, including the (α1→2) linkage in sucrose and kojibiose.[39] However, a BLAST analysis of these putative glucosidases of *L. acidophilus* ATCC 4356 and Alg3 showed very low similarity in protein sequence (between 24 and 31 %) (Table S2). The observed accumulation of kojibiose in the growth medium of *L. acidophilus* ATCC 4356 (Figure 6) thus may be due to lack of an extracellular α-

glucosidase active on (α1→2) linkages.Furthermore, it is possible that the α-glucosidase enzymes of this strain are only intracellular enzymes and that a suitable transporter for kojibiose is absent.

Hydrolytic activity of the *Bifidobacterium* strains on the GL34 mixture

Typically, bifidobacteria have extracellular endohydrolases acting on glycosidic bonds of oligo- and polymeric substrates, yielding smaller products which are internalized by carbohydrate-specific (ABC-type) transporters. Further utilization may be carried out by cytoplasmic GHs such as α/β-glucosidases and α/β-galactosidases to produce monosaccharides which are used for growth.[40'41] To try and identify the bifidobacterial enzymes responsible for utilization of GL34 compounds, cells grown in modified BM broth containing 5 mg mL^{-1} GL34 were harvested at 36 h (Figure 3). Three fractions were prepared, namely growth culture supernatants, concentrated culture supernatants and cytoplasmic extracts (see Methods). GL34 was incubated with concentrated culture supernatants and cytoplasmic extracts to verify the presence or absence of extra- and/or intracellular enzyme activities involved in degradation.

***Bifidobacterium adolescentis* ATCC 15703**

B. adolescentis was able to utilize all GL34 compounds, and only 10 and 60 % of F4 4′,2-glc-lac and F5 3′,2-glc-lac remained, respectively Figure 7-1). This also explains the relatively strong growth of this strain (Figure 3). None of the GL34 compounds were hydrolyzed by cell-free culture supernatants of this strain. Its cell extracts only hydrolyzed F1 4′-glc-lac, F2 2-glc-lac & F4 4′,2-glc-lac compounds (Figure 7-1). Three α-glucosidases are annotated in the *B. adolescentis* ATCC 15703 genome sequence (http://www.cazy.org), namely AglB; AglA and BAD-0971. AglB exhibited a preference for hydrolyzing (α1→4) linkages in maltose (van den Broek et al. 2003),[42] and most likely also is involved in cleavage of the (α1→4) linkages

present in the F1 and F4 compounds. AglA showed high hydrolytic activity only towards (α1→6) linkages in isomaltotriose and minor activity towards (α1→1) linkages in trehalose.[42] None of these two enzymes are able to degrade (α1→3) linkages occurring in F3 3′-glc-lac and F5 3′,2-glc-lac. It thus remained possible that during growth the F3 and F5 were (partially) degraded by the BAD-0971 enzyme, the third *B. adolescentis* ATCC 15703 α-glucosidase.

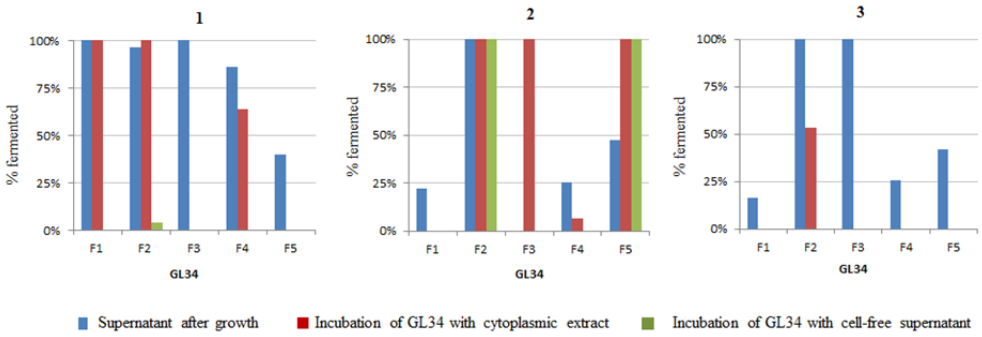


Figure 7: Consumption of GL34 compounds (%) during growth of 1) *B. adolescentis* ATCC 15703; 2) *B. longum* subsp. *infantis* ATCC 15697 and 3) *B. breve* DSM 20213, and after 24 h incubations of 5 mg mL^{-1} GL34 with cytoplasmic extracts or cell-free supernatants (obtained after growth of these three strains on the GL34 mixture).

The BAD-0971 protein has been annotated as an α-1,4-glucosidase but has not been characterized yet.[43] A BLASTP analysis was carried out to compare its protein sequence with those of the characterized glucosidases of *B. adolescentis* ATCC 15703 (AglA and AglB) and *B. breve* UCC 2003 (Agl1; Agl2 and Agl3). Agl3 from *B. breve* UCC 2003, as mentioned above, has a broad hydrolytic specificity towards (α1→2), (α1→3), (α1→4), (α1→5) and (α1→6) in kojibiose, turanose, maltose, leucrose and palatinose, respectively.[44] The two other α-glucosidases of this strain (Agl1 and Agl2) are more active in cleaving (α1→6) linkages (in panose, isomaltose, isomaltotriose, and palatinose) and less efficient in cleaving (α1→3) linkages in turanose and nigerose, (α1→4) in maltulose and (α1↔2) in sucrose.[45] AglA and Agl3 exhibit 59 % and 73 % similarity/identity, respectively, to the BAD-

0971 protein of *B. adolescentis* (Table S3). The BAD-0971 enzyme may have a broad hydrolytic specificity similar to Agl3. However F3 3′-glc-lac and F5 3′,2-glc-lac with (α1→3) linkages remained undigested in cell extracts. It cannot be excluded that the cell disruption process has weakened activity of BAD-0971 (annotated as an α-1,4-glucosidase), or other hitherto unidentified α-glucosidase enzymes.

***Bifidobacterium longum* subsp. *infantis* ATCC 15697**

The F2 2-glc-lac and F3 3′-glc-lac compounds were completely utilized during growth of *B. longum* subsp. *infantis*, and the F1 4′-glc-lac, F4 4′,2-glc-lac and F5 3′,2-glc-lac compounds only for 20-40% (Figure 7-2). Cell extracts only hydrolyzed F2 2-glc-lac, apparently involving (an) intracellular enzyme(s) (Figure 7-2). The F1 4′-glc-lac, F3 3′-glc-lac, F4 4′,2-glc-lac and F5 3′,2-glc-lac structures were not utilized by cell extracts of this strain, suggesting that it lacks α-glucosidase enzymes acting on glucose (α1→3)/(α1→4) linked to galactosyl residue. The complete use of the F3 3′-glc-lac compound during growth of *B. longum* subsp. *infantis* ATCC 15697 thus remained unexplained again. No extracellular activity of *B. longum* subsp. *infantis* toward the GL34 mixture was detected (Figure 7-2). Its limited activity on the GL34 mixture resulted in the lowest growth extent (37 %) compared to the other two bifidobacteria (Table 1). Various glucosidases have been annotated in the genome of *B. longum* subsp. *infantis* mainly with activity on (α1→4) and (α1→6) glucosidic linkages, namely α-1,4 glucosidase (locus tag BLIJ-0129); putative amylase (locus tag BLIJ-0286); putative iso-amylase (locus tag BLIJ-0286 and BLIJ-2315) and oligo-1,6-glucosidase (locus tag BLIJ-2526) (www.cazy.org/). However, BLAST analysis showed very low similarity in their protein sequence between the annotated glucosidases from this strain and Alg3 (Table S4). This most likely explains the poor growth of this strain on the GL34 mixture, and its limited intracellular activity on (α1→3)/(α1→4) glucosidic linkages in F1 4′-glc-lac, F3 3′-glc-lac, F4 4′,2-glc-lac and F5 3′,2-glc-lac.

***Bifidobacterium breve* DSM 20213**

In case of *B. breve,* four of the five GL34 compounds (F1 4′-glc-lac, F3 3′-glc-lac, F4 4′,2-glc-lac and F5 3′,2-glc-lac) (partly) remained unutilized in culture supernatants after growth (Figure 7-3). Amongst the three bifidobacteria tested only *B. breve* possessed extracellular activity on the mixture GL34: the F2 2-glc-lac and F5 3′,2-glc-lac compounds were completely degraded by cell-free supernatants after 24 h incubation (Figure 7-3). The extracellular activity was also observed previously in different *B. breve* strains, however, on (α1→4) and (α1→6) glucosidic linkages present in starch, amylopectin and pullulan.[46] Nevertheless, cell extracts fully hydrolyzed F2 2-glc-lac, F3 3′-glc-lac and F5 3′,2-glc-lac (Figure 7-3). Most likely *B. breve* is unable to transport F3 3′-glc-lac and F5 3′,2-glc-lac into the cell. These data suggest that intracellular enzymes of this strain were able to cleave off the glucose units (α1→3) linked to the galactosyl residues occurring in F3 3′-glc-lac and F5 3′,2-glc-lac but not the glucose units (α1→4) linked to the galactosyl residues in F1 4′-glc-lac and F4 4′,2-glc-lac.

The putative α-glucosidases in the *B. breve* DSM 20213 genome, however, are annotated to act mainly on (α1→4) and (α1→6) glucosidic linkages; namely α-1,4 glucosidase (locus tag BBBR-0095); putative amylase (locus tags BBBR-0101; BBBR-0825 and BBBR-0257) and oligo-1,6-glucosidase (locus tags BBBR-0484 and BBBR-1863) (www.cazy.org/). This uncharacterized α-1,4 glucosidase (locus tag BBBR-0095) was found to have 99 % similarity with Alg3 of *B. breve* UCC 2003 (Table S4). This annotated α-1,4 glucosidase thus also may be able to hydrolyze (α1→3) linkage in F3 3′-glc-lac and F5 3′,2-glc-lac. Structures of F1 4′-glc-lac and F4 4′,2-glc-lac may be inaccessible to this α-glucosidase, or it is unable to cleave their (α1→4) linkages, thus explaining their (very) limited degradation by *B. breve* DSM 20213.

The annotated β-galactosidases (family GH2) of these three *Bifidobacterium* strains are active on the F2 2-glc-lac compound, containing a galactosyl-moiety with a (β1→4) linkage to kojibiose. Following growth on GL34 as only carbon source, all three strains exhibited clear intracellular activity with F2 2-glc-lac. The data showed that individual *Bifidobacterium* strains have preference for degradation of glucosylated lactose compounds with (α1→3) and (α1→4) glucosidic linkages.

DISCUSSION

The GL34 compounds generally consist of a lactose molecule at the reducing end that is elongated with one or more glucose molecules involving different linkage types.[25] This combination of different monomers, i.e. glucose and galactose, with various linkage types ((α1→2), (α1→3), (α1→4) and (β1→4)) and different degrees of polymerization, increases the diversity of lactose-derived oligosaccharides available. The GL34 compounds were resistant to degradation by the α-amylases of porcine pancreas and *Aspergillus oryzae*. These are endo-acting enzymes (EC 3.2.1.1), degrading (α1→4) glucosidic linkages in polysaccharides such as starch or glycogen,[47] mainly yielding glucose and maltose. Also various MOS-acting enzymes were tested: The α-glucosidase enzyme of yeast used is known as an exo-acting enzyme, hydrolyzing (α1→4) glucosidic linkages but only at the terminal non-reducing (1→4)-linked α-glucose residues of di- and oligosaccharides to release a single glucose unit.[48] Iso-amylase from *Pseudomonas* sp. is an (α1→4,6) debranching enzyme.[47] Pullulanase type I of *K. planticola* hydrolyzes (α1→6) glucosidic linkages in pullulan and starch.[49] Also these 3 enzymes failed to cleave any compounds in the GL34 mixture. Incubations with β-galactosidase enzymes however did result in hydrolysis, but only the F2 2-glc-lac molecule disappeared. This is explained by the ability of these enzymes to catalyze hydrolysis of β-glycosidic bonds between galactose and its organic moiety. The combined data thus

shows that the GL34 compounds are (largely) resistant to hydrolysis by these common carbohydrate degrading enzymes (Figure 1).

There is abundant clinical evidence for the important roles of *Bifidobacterium* and *Lactobacillus* species in the eco-physiology of the intestinal microbiota,[50,51] and different strains of lactobacilli are marketed as commercial probiotics. Individual bifidobacteria are known to have specific substrate preferences.[52] The GL34 mixture stimulated growth of bifidobacteria, but indeed to different extents. Only utilization of the F2 2-glc-lac compound has been studied previously and was shown to have a limited stimulatory effect on the growth of *B. breve* 26M2. This F2 compound did not stimulate growth of lactobacilli tested in our present study, as previously shown for *L. casei* LC-01.[24] However, we observed that F2 2-glc-lac stimulated growth of the probiotic bacteria *L. reuteri* 121, *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis* ATCC 15697, *B. breve* DSM 20213, and also of two commensal bacteria, *E. coli* Nissle and *B. thetaiotaomicron*, albeit to various extents. This F2 compound thus is less selective in comparison with the other compounds in the GL34 mixture. Both F1 4′-glc-lac and F4 4′,2-glc-lac also stimulated growth of all three tested bifidobacteria, again to various extents. The presence of an (α1→3) linkage makes F3 3′-glc-lac more selective than F1 and F4: F3 was utilized by only two out of three studied *Bifidobacterium* strains, *B. adolescentis* ATCC 15703 and *B. breve* DSM 20213. Also F5 3′,2-glc-lac with both (α1→2) and (α1→3) glucosidic linkages showed similar stimulatory effects on all three *Bifidobacterium* strains.

In conclusion, the GL34 mixture promotes growth of the tested bacteria to different extents. The bifidobacteria tested generally were better at degrading GL34 compounds than the lactobacilli and commensal bacteria. The stronger metabolic toolset of bifidobacteria in comparison with lactobacilli also has been observed when comparing their growth on human milk oligosaccharides and other prebiotic oligosaccharides as primary carbon source.[28,44,53,54] The GL34 mixture thus showed

potential to shift microbiota composition by specifically stimulating growth of bifidobacteria, particularly *B. adolescentis*.

Four out of five compounds in this GL34 mixture exerted high and selective growth stimulatory effects towards health-beneficial probiotic bifidobacteria. The combination of monomer composition and linkage type clearly determines the fermentable properties of the GL34 compounds. Individual gut bacteria were able to utilize only specific compounds in the GL34 mixture. Synergistic activities between bacterial species thus are likely to be essential for the utilization of the whole GL34 mixture. In future work this will be studied in more detail e.g. by using faecal bacterial cultures. Only *B. adolescentis* was able to utilize almost all structures, providing a potential synbiotic combination.

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REFERENCES

1. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 2006;124(4):837-848.
2. Flint HJ, Duncan SH, Scott KP, Louis P. Interactions and competition within the microbial community of the human colon: Links between diet and health: Minireview. *Environ Microbiol*. 2007;9(5):1101-1111.
3. Louis P, Scott KP, Duncan SH, Flint HJ. Understanding the effects of diet on bacterial metabolism in the large intestine. *J Appl Microbiol.*Vol 102.; 2007:1197-1208.
4. Gibson GR, Hutkins R, Sander ME, Prescott SL, Reimer RA, Salminen SJ, Scott K., Stanton C, Swanson KS, Cani PD, Verbeke K, Reid G. Expert consensus document: The international scientific association for probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol*. 2017;14(8):491-502.
5 Bindels LB, Delzenne NM, Cani PD, Walter J. Towards a comprehensive concept for prebiotics. *Nat Rev Gastroenterol Hepatol* 2015;12**:** 303–310
6 Yan YL, Hu Y, Gänzle MG. Prebiotics, FODMAPs and dietary fiber — conflicting concepts in development of functional food products?. *Curr Opin Food Sci* 2018;20**:** 30–37
7. Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H, Stahl B, Guarner F, Respondek F, Whelan K, Coxam V, Davicco M-Jeanne, Léotoing L, Wittrant Y, Delzenne NM, Cani PD, Neyrinck AM, Meheust A. Prebiotic effects: metabolic and health benefits. *Br J Nutr*. 2010;104 Suppl(November):S1-63.
8. Callaway TR, Edrington TS, Anderson RC, Harvey RB, Genovese KJ, Kennedy CN, Venn DW, Nisbet DJ. Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. *Anim Heal Res Rev*. 2008;9(2):217-225.
9. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology* 2012;22(9):1147-1162.
10. Slavin J. Fiber and prebiotics: Mechanisms and health benefits. *Nutrients* 2013;5(4):1417-1435.
11. Macfarlane GT, Steed H, Macfarlane S. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J Appl Microbiol*. 2008;104(2):305-344.
12 Ferreira-Lazarte A, Olano A, Villamiel M, Moreno FJ. Assessment of in vitro digestibility of dietary carbohydrates using rat small intestinal extract. *J. Agric. Food Chem*. 2017;65: 8046–8053
13. Bai Y, Böger M, Van Der Kaaij RM, Woortman AJJ, Pijning T, van Leeuwen SS, van Bueren AL, Dijkhuizen L. *Lactobacillus reuteri* strains convert starch and

maltodextrins into homoexopolysaccharides using an extracellular and cell-associated 4,6-α-glucanotransferase. *J Agric Food Chem*. 2016;64(14):2941-2952.
14. Rycroft CE, Jones MR, Gibson GR, Rastall RA. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *J Appl Microbiol*. 2001;91(5):878-887.
15. Marquina D, Santos A, Corpas I, Muñoz J, Zazo J, Peinado JM. Dietary influence of kefir on microbial activities in the mouse bowel. *Lett Appl Microbiol*. 2002;35(2):136-140.
16. Lehmann U, Robin F. Slowly digestible starch - its structure and health implications: a review. *Trends Food Sci Technol*. 2007;18(7):346-355.
17. Jaskari J, Kontula P, Siitonen A, Jousimies-Somer H, Mattila-Sandholm T, Poutanen K. Oat α-glucan and xylan hydrolysates as selective substrates for *Bifidobacterium* and *Lactobacillus* strains. *Appl Microbiol Biotechnol*. 1998;49(2):175-181.
18. Pandey KR, Naik SR, Vakil B V. Probiotics, prebiotics and synbiotics- a review. *J Food Sci Technol*. 2015;52(12):7577-7587.
19. Rastall RA, Maitin V. Prebiotics and synbiotics: Towards the next generation. *Curr Opin Biotechnol*. 2002;13(5):490-496.
20. van Leeuwen SS, Kuipers BJH, Dijkhuizen L, Kamerling JP. Comparative structural characterization of 7 commercial galacto-oligosaccharide (GOS) products. *Carbohydr Res*. 2016;425:48-58.
21. Boehm G, Moro G. Structural and functional aspects of prebiotics used in infant nutrition. *J Nutr*. 2008;138(9):1818S-1828S.
22. Rijnierse A, Jeurink P V., Van Esch BCAM, Garssen J, Knippels LMJ. Food-derived oligosaccharides exhibit pharmaceutical properties. *Eur J Pharmacol*.Vol 668.; 2011.
23. Ohkusa T, Ozaki Y, Sato C, Mikuni K, Ikeda H. Long-term ingestion of lactosucrose increases *Bifidobacterium* sp. in human fecal flora. *Digestion* 1995;56(5):415-420.
24. García-Cayuela T, Díez-Municio M, Herrero M, Martínez-Cuesta MC, Peláez C, Requena T, Moreno FJ. Selective fermentation of potential prebiotic lactose-derived oligosaccharides by probiotic bacteria. *Int Dairy J*. 2014;38(1):11-15.
25. Pham TTH, Dijkhuizen L, van Leeuwen SS. Structural characterization of glucosylated lactose derivatives synthesized by the *Lactobacillus reuteri* GtfA and Gtf180 glucansucrase enzymes. *Carbohydr Res*. 2017;449:59-64.
26. Lammerts van Bueren A, Mulder M, Leeuwen S van, Dijkhuizen L. Prebiotic galacto-oligosaccharides activate mucin and pectic galactan utilization pathways in the human gut symbiont *Bacteroides thetaiotaomicron*. *Sci Rep*. 2017;7:40478.
27. Daniels L, Zeikus JG. Improved culture flask for obligate anaerobes. *Appl Microbiol*. 1975;29(5):710-711.
28. Watson D, O'Connell Motherway M, Schoterman MHC, van Neerven RJJ, Nauta A, Van Sinderen D. Selective carbohydrate utilization by lactobacilli and bifidobacteria.

J Appl Microbiol. 2013;114(4):1132-1146.

29. Koropatkin NM, Martens EC, Gordon JI, Smith TJ. Starch catabolism by a prominent human gut symbiont is directed by the recognition of amylose helices. *Structure* 2008;16(7):1105-1115.
30. Sambrook J, W Russell D. Molecular cloning: A laboratory manual. *Cold Spring Harb Lab Press Cold Spring Harb NY*. 2001:999.
32. Díez-Municio M, Montilla A, Jimeno ML, Corzo N, Olano A, Moreno FJ. Synthesis and characterization of a potential prebiotic trisaccharide from cheese whey permeate and sucrose by *Leuconostoc mesenteroides* dextransucrase. *J Agric Food Chem*. 2012;60:1945-1953.
32. Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chiang HC, Hooper LV, Gordon JI. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science* 2003;299(5615):2074-2076.
33. Mason TG, Richardson G. *Escherichia coli* and the human gut: some ecological considerations. *J Appl Bacteriol*. 1981;51(1):1-16.
34. Gangoiti J, Meng X, Lammerts van Bueren A, Dijkhuizen L. Draft genome sequence of *Lactobacillus reuteri* 121, a Source of α-glucan and β-fructan exopolysaccharides. *Genome Announc*. 2017;5(10):e01691-16.
35. Ganzle MG, Follador R. Metabolism of oligosaccharides and starch in lactobacilli: A review. *Front Microbiol*. 2012;3(SEP):1-15.
36. Pan Q, Zhu J, Liu L, Cong Y, Hu F, Li J, Yu X. Functional identification of a putative β-galactosidase gene in the special lac gene cluster of *Lactobacillus acidophilus*. *Curr Microbiol*. 2010;60(3):172-178.
37. Schwab C, Sørensen KI, Gänzle MG. Heterologous expression of glycoside hydrolase family 2 and 42 β-galactosidases of lactic acid bacteria in *Lactococcus lactis*. *Syst Appl Microbiol*. 2010;33(6):300-307.
38. Palomino MM, Allievi MC, Fina Martin J, Waehner PM, Prado AM, Sanchez RC, Ruzal SM. Draft genome sequence of the probiotic strain *Lactobacillus acidophilus* ATCC 4356. *Genome Annoucements* 2015;3(1):7-8.
39. Kelly ED, Bottacini F, O'Callaghan J, O'Connell Motherway M, O'Connell KJ, Stanton C, van Sinderen D. Glycoside hydrolase family 13 α-glucosidases encoded by *Bifidobacterium breve* UCC2003; A comparative analysis of function, structure and phylogeny. *Int J Food Microbiol*. 2016;224:55-65.
40. O'Connell Motherway M, Fitzgerald GF, van Sinderen D. Metabolism of a plant derived galactose-containing polysaccharide by *Bifidobacterium breve* UCC2003. *Microb Biotechnol*. 2011;4(3):403-416.
41. O'Connell KJ, O'Connell-Motherway M, O'Callaghan J, Fitzgerald GF, Ross RP, Ventura M, Stanton C, van Sinderen D. Metabolism of four α-glycosidic linkage-containing oligosaccharides by *Bifidobacterium breve* UCC2003. *Appl Environ Microbiol*. 2013;79(20):6280-6292.

42. van den Broek L a M, Struijs K, Verdoes JC, Beldman G, Voragen a GJ. Cloning and characterization of two alpha-glucosidases from *Bifidobacterium adolescentis* DSM20083. *Appl Microbiol Biotechnol*. 2003;61(1):55-60.
43. Suzuki, T., Tsuda, Y., Kanou, N., Inoue, T., Kumazaki, K., Nagano S, Hirai, S., Tanaka, K. and Watanabe K. *Bifidobacterium adolescentis* complete genome sequence. *Publ Only Database*. 2006.
44. McLaughlin HP, Motherway MOC, Lakshminarayanan B, Stanton C, Ross RP, Brulc J, Menon R, O'Toole PW, van Sinderen D. Carbohydrate catabolic diversity of bifidobacteria and lactobacilli of human origin. *Int J Food Microbiol*. 2015;203:109-121.
45. Pokusaeva K, O'Connell-Motherway M, Zomer A, Fitzgerald GF, Van Sinderen D. Characterization of two novel α-glucosidases from *Bifidobacterium breve* UCC2003. *Appl Environ Microbiol*. 2009;75(4):1135-1143.
46. Ryan SM, Fitzgerald GF, van Sinderen D. Screening for and identification of starch-, amylopectin-, and pullulan-degrading activities in bifidobacterial strains. *Appl Environ Microbiol*. 2006;72(8):5289-5296.
47. van der Maarel MJEC, van der Veen B, Uitdehaag JCM, Leemhuis H, Dijkhuizen L. Properties and applications of starch-converting enzymes of the alpha-amylase family. *J Biotechnol*. 2002;94(2):137-155.
48. Reese ET, Maguire AH, Parrish FW. Glucosidases and exo-glucanases. *Can J Biochem*. 1968;46(1):25-34.
49. Domań-Pytka M, Bardowski J. Pullulan degrading enzymes of bacterial origin. *Crit Rev Microbiol*. 2004;30:107-121.
50. Picard C, Fioramonti J, Francois A, Robinson T, Neant F, Matuchansky C. Review article: Bifidobacteria as probiotic agents - Physiological effects and clinical benefits. *Aliment Pharmacol Ther*. 2005;22(6):495-512.
51. Sanders ME, Klaenhammer TR. Invited review: the scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. *J Dairy Sci*. 2001;84(2):319-331.
52. Degnan B a., Macfarlane GT. Comparison of carbohydrate substrate preference in eight species of bifidobacteria. *FEMS Microbiol Lett*. 1991;84:151-156.
53. González R, Klaassens ES, Mahnen E, De Vos WM, Vaughan EE. Differential transcriptional response of *Bifidobacterium longum* to human milk, formula milk, and galacto-oligosaccharide. *Appl Environ Microbiol*.Vol 74.; 2008:4686-4694.
54. Sela DA, Chapman J, Adeuya A, Kim JH, Chen F, Whitehead TR, Lapidus A, Rokhsar DS, Lebrilla CB, German JB, Price NP, Richardson PM, Mills DA. The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. *Proc Natl Acad Sci U S A*. 2008;105(48):18964-18969.

Supplemental data

Table S1: Carbohydrate acting enzymes used to analyze degradation of the GL34 compounds. The pH and temperature values used are shown.

Enzymes	Origins	Source	GH family	Reaction conditions	
				pH	Temperature °C
α-amylase 1	Porcine pancreas	Sigma-Aldrich	GH13	7.0	50
α-amylase 2	*Aspergillus oryzae*	Megazyme	GH13	5.0	50
α-glucosidase	Yeast	Megazyme	GH13	7.0	40
Iso-amylase	*Pseudomonas sp.*	Megazyme	GH13	4.0	40
Pullulanase type 1	*Klebsiella planticola*	Megazyme	GH13	5.0	40
β-galactosidase 1	*Aspergillus oryzae*	Megazyme	GH2	4.7	45
β-galactosidase 2	*Kluyveromyces lactis*	Megazyme	GH2	7.0	40

Table S2: Sequence similarity levels between Agl3 from *Bifidobacterium breve* UCC 2003 and putative α-glucosidases encoded in the *Lactobacillus acidophilus* ATCC 4356 genome.

NCBI accession number	Annotated GH family*	Query cover (%)	Identity level (%)
WP_003546237.1	GH31	35	23
WP_003548000.1	GH31	28	24
WP_003548741.1	GH31	21	23
WP_003549626.1	GH13	40	26
WP_003549917.1	GH13 GH31	28	30
WP_011254098.1	GH13 GH31	93	29
WP_011254225.1	GH13	46	26
WP_011254315.1	GH13	78	31
WP_011254601.1	GH13 GH31	96	28
WP_021721607.1	GH31	25	24

* Annotation of proteins from the genome sequence of *L. acidophilus* ATCC 4356 was carried out using dbCAN (http://csbl.bmb.uga.edu/dbCAN).

Table S3: Protein sequence similarity levels of BAD-0971 from *B. adolescentis* ATCC 15703 to annotated α-glucosidases in other bifidobacteria.

Protein name	Annotated or proven functions	Bacterial strain	NCBI accession number	Identity level (%)
AglA	α-1,6-glucosidase	*B. adolescentis* ATCC 15703	Query_123011	73
AglB	α-glucosidase	*B. adolescentis* ATCC 15703	Query_123011	73
Agl1	α-1,6-glucosidase	*B. breve* UCC 2003	Query_83439	30
Agl2	α-1,6-glucosidase	*B. breve* UCC 2003	Query_98509	32
Agl3	α-glucosidase	*B. breve* UCC 2003	Query_165455	59

Table S4: Similarity levels of GH13 glucosidase proteins from *B. longum* subsp. *infantis* ATCC 15697 and *B. breve* DSM 20123 to the annotated Alg3 from *B. breve* UCC 2003.

Bacterial strain	Locus tags	Annotated or proven functions	NCBI accession number	Identity level (%)
B. infantis ATCC 15697	BLIJ-0129	α-1,4-glucosidase	Query_130701	19
B. infantis ATCC 15697	BLIJ-0286	putative amylase	Query_56433	16
B. infantis ATCC 15697	BLIJ-1799	putative isoamylase	Query_179069	17
B. infantis ATCC 15697	BLIJ-2315	putative isoamylase	Query_117079	16
B. infantis ATCC 15697	BLIJ-2526	oligo-1,6-glucosidase	Query_140967	29
B. breve DSM 20123	BBBR-0095	α-1,4-glucosidase	Query_5141	99
B. breve DSM 20123	BBBR-0101	putative amylase	Query_163329	25
B. breve DSM 20123	BBBR-0257	putative amylase	Query_188975	17
B. breve DSM 20123	BBBR-0484	Oligo-1,6-glucosidase	Query_211369	29
B. breve DSM 20123	BBBR-0825	putative amylase	Query_229645	19
B. breve DSM 20123	BBBR-1863	Oligo-1,6-glucosidase	Query_8587	28

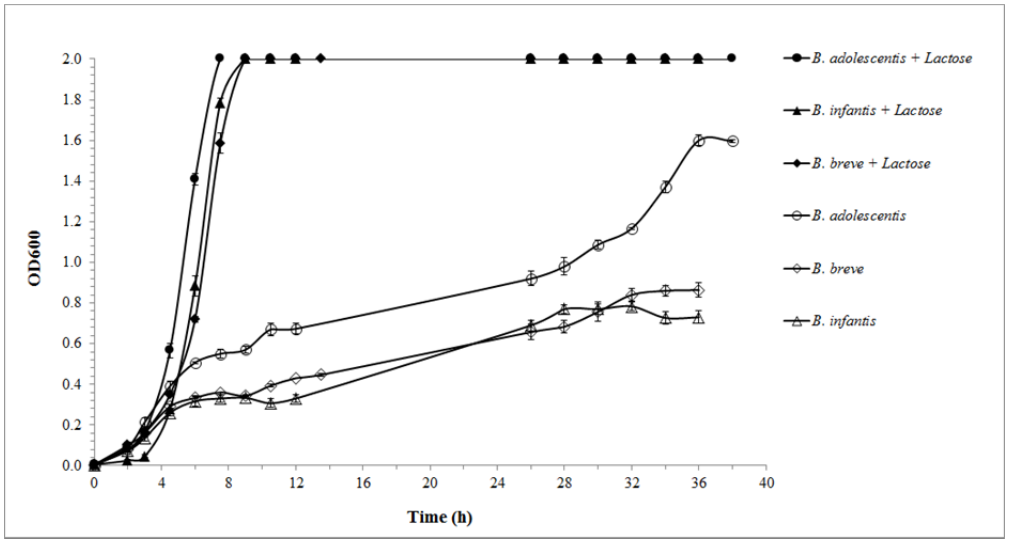

Figure S1: Growth of *B. adolescentis* ATCC 15703, *B. infantis* ATCC 15697 and *B. breve* DSM 20123 on GL34 compounds (5 mg mL^{-1}). Lactose (5 mg mL^{-1}) served as positive control, growth studies were carried out in triplicate.